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Distal-less and *homothorax* regulate multiple targets to pattern the *Drosophila* antenna

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SUMMARY

The *Drosophila* antenna is a highly derived appendage required for a variety of sensory functions including olfaction and audition. To investigate how this complex structure is patterned, we examine the specific functions of genes required for antenna development. The nuclear factors, Homothorax, Distal-less and Spineless, are each required for particular aspects of antennal fate. Coexpression of Homothorax, necessary for nuclear localization of its ubiquitously expressed partner Extradenticle, with Distal-less is required to establish antenna fate. Here we test which antenna patterning genes are targets of Homothorax, Distal-less and/or Spineless. We report that the antennal expression of *dachshund*, *atonal*, *spalt*, and *cut* requires Homothorax and/or Distal-less, but not Spineless. We conclude that Distal-less and Homothorax specify antenna fates via regulation of multiple genes. We also report for the first time phenotypic consequences of losing either *dachshund* or *spalt* and *spalt-related* from the antenna. We find that *dachshund* and *spalt*/*spalt-related* are essential for proper joint formation between particular antennal segments. Furthermore, the *spalt*/*spalt-related* null antennae are defective in hearing. Hearing defects are also associated with the human diseases Split Hand/Split Foot Malformation and Townes-Brocks Syndrome, which are linked to human homologs of *Distal-less* and *spalt*, respectively. We therefore propose that there are significant genetic similarities between the auditory organs of humans and flies.

Key words: *Distal-less*, *extradenticle*, *homothorax*, *atonal*, *cut*, *dachshund*, *spalt*, *spalt-related*, *spineless*, Antenna, Audition, *Drosophila*, Townes-Brocks syndrome, Split Hand/Split Foot Malformation

INTRODUCTION

The adult fruit fly antenna consists of six segments. From proximal to distal, these are called a1, a2, a3, a4, a5 and arista (a6; Fig. 1A). The different segments of the antenna serve distinct sets of functions including hygrosensation (arista), thermosensation (a3), olfaction (a3), and audition (arista and a2) (Carlson, 1996; Eberl, 1999; Goepfert and Robert, 2001; Sayeed and Benzer, 1996). Although the various functions of the antenna have been studied in detail, there is relatively little known about the genetic hierarchy that governs antenna formation. We are investigating what genes are involved and how they are involved in patterning the *Drosophila* antenna to understand how particular peripheral sensory structures are generated along the proximodistal (PD) axis of the antenna.

In the antenna, as in other appendages, the process of pattern formation requires both limb fate information and PD information. The homeodomain transcription factor-encoding genes, *Distal-less* (*Dll*) and *homothorax* (*hth*), provide both. Losing the function of either *Dll* or *hth*, results in deletions of the distal and proximal domains, respectively (Casares and Mann, 1998; Cohen and Jurgens, 1989; Pai et al., 1998). In addition, coexpression of *Dll* and *hth* is required for the specification of antenna fate (Dong et al., 2000). Losing the function of either gene results in antenna to leg transformation (Fig. 1B,C) (Casares and Mann, 1998; Cohen and Jurgens, 1989; Dong et al., 2000; Sato, 1984; Sunkel and Whittle, 1987).

One might expect the targets of *Dll* or *hth* for PD patterning to be expressed in both the antenna and the leg. Consistent with this, the *Dll* targets, *bric a brac* (*bab*), *aristaless* (*al*), and *BarH1/BarH2*, are expressed and required in both the distal antenna and leg (Campbell and Tomlinson, 1998; Godt et al., 1993; Kojima et al., 2000; Schneitz et al., 1993). In contrast, the targets of *Dll* and *hth* involved in antenna specification would be predicted to have antenna-specific patterns of expression. Consistent with this, *spalt* (*sal*), a known *Dll* and *hth* target (Dong et al., 2000), and *spalt-related* (*salr*; adjacent and homologous genes with similar expression patterns) are expressed in identical circular patterns in the antennal disc (Barrio et al., 1999; Wagner-Bernholz et al., 1991) and only expressed at low levels in the leg imaginal disc, proximal to the presumptive leg (Fig. 3G). Another target is a bHLH-PAS encoding gene, *spineless* (*ss*), a homolog of the mammalian dioxin receptor gene (Duncan et al., 1998). As with *Dll* and *hth* loss-of-function mutants, loss of *ss* also results in antenna to leg transformations (Balkaschina, 1929; Burgess and Duncan, 1990; Struhl, 1982) (Fig. 1D). *Dll* is required for the antennal expression of *ss* (Duncan et al., 1998). Also, loss of

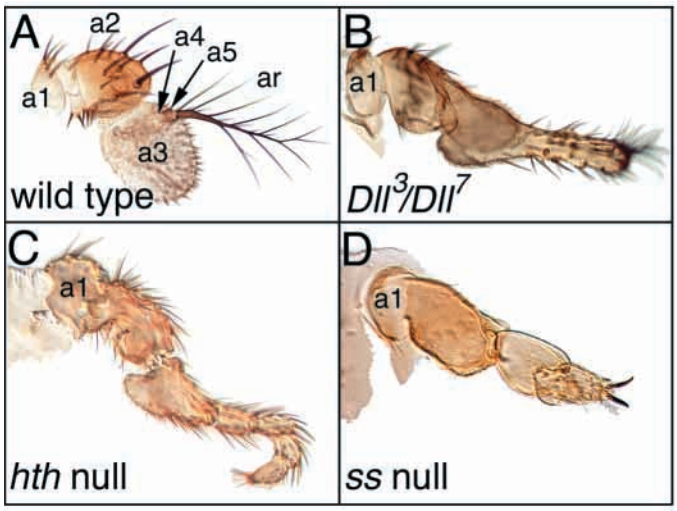

Fig. 1. *Dll*, *hth* and *ss* mutants all exhibit antenna to leg transformations. (A) Wild-type antenna. (B) Dll^{3}/Dll^{7} hypomorphic antenna in which distal a3 and the arista are transformed toward leg. (C) A large hth^{P2} clone in the antenna results in transformation of a1 to arista into leg structures. (D) *ss* null antenna of genotype $Df(3R)ss^{D114.4}/ss^{D114.9}$. a1-a5, antennal segments 1-5: ar, arista.

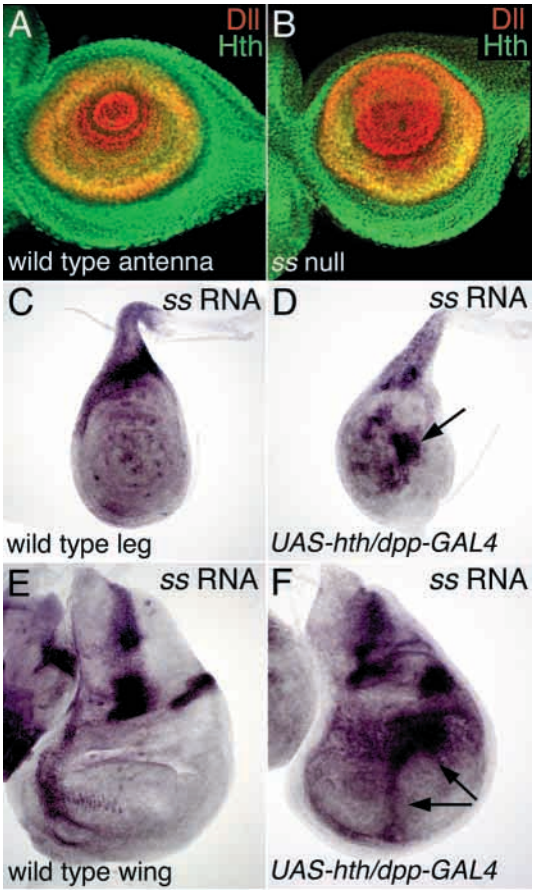

Fig. 2. *ss* is regulated by *Dll* and *hth*. (A) Wild type antenna disc showing Hth protein (green) and Dll protein (red). (B) *ss* null antenna disc of genotype $Df(3R)ss^{D114.4}/ss^{D114.9}$ in which Hth and Dll expression is normal. (C) Wild-type expression of *ss* in a late third instar leg disc. (D) Expression of *ss* is induced in the distal leg (arrow) by ectopic expression of Hth. (E) Wild-type expression of *ss* in a late third instar wing disc. (F) Expression of *ss* is induced in the wing pouch (arrows) by ectopic expression of Hth.

hth in the antenna results in loss of *ss* expression (Ian Duncan, personal communication). This, in conjunction with the *ss* transformation phenotype, raised the possibility that *ss* is a primary target through which *Dll* and *hth* effect antenna specification. That both *Dll* and *hth* mutants exhibit PD defects in the antenna in addition to transformation phenotypes, while *ss* leads primarily to transformation, indicated that *Dll* and *hth* almost certainly have functions that are not mediated by *ss*. We therefore focussed on genes with antenna-specific patterns of expression and tested whether these were regulated by *ss*. We demonstrate here that there are multiple targets of both Dll and Hth in the antenna whose activation is independent of Ss.

In particular, we report that nuclear factors, *dachshund* (*dac*), *atonal* (*ato*), *sal* and *cut* (*ct*) are antenna-specific targets of *Dll* and/or *hth*. In addition, through phenotypic analyses, we identify novel functions for *dac* in the antenna and for *sal* and *salr* in both antenna and eye. Furthermore although *ss* is required for antennal specification, the antennal expression of *dac*, *ato*, *sal*, and *ct* is independent of *ss*. The work we present here defines distinct roles for *Dll*, *hth* and *ss* in antenna specification. With a greater understanding of how the antenna is specified and its PD axis is subdivided, we can now begin to explain how key sensory structures are both fated and positioned during antennal development.

MATERIALS AND METHODS

Fly strains and genetic manipulations

The following fly strains were employed: (1) *DllGAL4/T(2:3) SM6a; TM6B*; (2) *Dll3/T(2:3) SM6a; TM6B*; (3) *FRT42D DllSA1* (Gorfinkiel et al., 1997); (4) *y w FLPase; FRT82B M(3)121 πM/ TM3, Ser*; (5) *w; FRT82B hthP2/TM6B, Tb, Hu* (Pai et al., 1998); (6) *Df(3R)ssD114.4/TM6* (Ian Duncan); (7) *ssD114.9/TM6* (Ian Duncan); (8) *ssD115.7/TM6* (Ian Duncan); (9) *w; dac4 FRT40A/T(2:3) SM6a; TM6B*; (10) *w; dac3 FRT40A/T(2:3) SM6a; TM6B*; (11) *dac-lacZ/T(2:3) SM6a; TM6B* (Graeme Mardon); (12) *ato1* (Jarman et al., 1994; Jarman et al., 1995); (13) *sal16 FRT40A* (Ethan Bier); (14) *salFCK–25/T(2:3) SM6a; TM6B* (Rosa Barrio); (15) *w; Df(2L)32FP-5 FRT40A/T(2:3) SM6a; TM6B* (Barrio et al., 1999); (16) *dpp-GAL4 (A.3)/TM6B* (Morimura et al., 1996); (17) *w; UAS-GFP-hth8/TM6B, Tb, Hu* (Casares and Mann, 1998); (18) *y hs-FLPase; FRT82B 2piM*; (19) *y w ey-FLPase GMR-lacZ; FRT40A*; and (20) *y w ey-FLPase GMR-lacZ; FRT42D*.

Dll, *hth*, and *sal/salr* null clones were generated using the FLP/FRT system (Xu and Rubin, 1993). Animals of the genotype: *y hs-FLPase; FRT82B 2piM/FRT82B* hth^{P2} were heatshocked at 37°C for 1 hour at 48-72 hours after egg laying (AEL) and examined in mid- to late-third instar. *Dll* and *sal/salr* null clones were generated using *ey-FLPase* (Newsome et al., 2000). The genotypes of the larvae and adults examined were: *y w ey-FLPase GMR-lacZ; FRT42D 2piM/FRT42D* Dll^{SA1} and *y w ey-FLPase GMR-lacZ; FRT40A/Df(2L)32FP-5 FRT40A*. In addition to *Dll* null clones, strong *Dll* hypomorpic antennae of the genotype Dll^{GAL4}/Dll^{3} were examined. These alleles were balanced over *T(2:3) SM6a; TM6B, Hu, Tb*. *Dll* mutant larvae therefore could be identified by lack of a *Tb* phenotype.

The *ss* null genotype examined was $Df(3R)ss^{D114.4}/ss^{D114.9}$. Strong *ss* hypomorphs of the genotype $Df(3R)ss^{D114.4}/ss^{D115.7}$ also were examined and exhibited similar phenotypes. *dac* null animals were

generated by crossing *w; dac^{4} FRT40A/T(2:3) SM6a; TM6B* with *w; dac^{3} FRT40A/T(2:3) SM6a; TM6B*. Null animals were selected on the basis of the absence of the *Tubby* phenotype. The *ato* nulls tested were homozygous for *ato^{1}*, which is homozygous viable.

In addition to *sal/salr* null clones, two additional genotypes lacking *sal* and *salr* in the antenna also were examined: *sal$^{FCK–25}$/sal$^{FCK–25}$* and *sal$^{FCK–25}$/Df(2L32FP-5 FRT40A*. *sal$^{FCK–25}$* flies have a small deletion of *sal/salr* regulatory sequences that results in the loss of both *sal* and *salr* expression in the antenna (Barrio et al., 1999). *Df(2L)32FP-5* is a deletion that removes both the *sal* and *salr* genes (Barrio et al., 1999). Since both *sal$^{FCK–25}$* and *Df(2L)32FP-5 FRT40A* were balanced over *T(2:3) SM6a; TM6B*, *sal* mutants could be identified by lack of the *Tubby* phenotype.

Ectopic expression of *hth* was induced using the GAL4/UAS binary system (Brand and Perrimon, 1993). *dpp-GAL4* was used to activate *UAS-GFP-hth* along the anterior-posterior compartment boundary of the developing imaginal discs.

In situ hybridization and immunohistochemistry

In situ hybridization was carried out as previously described (Jiang et al., 1991; Tautz and Pfeifle, 1989). Antibody stainings and immunohistochemistry also were carried out as described previously (Halder et al., 1998). Antibodies used were: rabbit anti-Hth (Pai et al., 1998), rabbit anti-Dll (Panganiban et al., 1995), mouse anti-Dll (Vachon et al., 1992), rat anti-Sal (Barrio et al., 1999), rabbit anti-Atonal (Jarman et al., 1995), mouse anti-Cut and mouse anti-Dac (both from the University of Iowa Developmental Studies Hybridoma Bank). Secondary antibodies coupled to Cy2, Cy3, and Cy5 were obtained from Jackson ImmunoResearch. Imaging was carried out on a BioRad MRC1024 confocal microscopy and a Zeiss Axioplan microscope equipped with an Axiocam.

RESULTS

spineless acts downstream of both *Distal-less* and *homothorax*

As with *Dll* and *hth* loss-of-function mutants, loss of *ss* also results in antenna to leg transformations (Fig. 1D) (Balkaschina, 1929; Burgess and Duncan, 1990; Struhl, 1982). This led us to investigate the genetic relationship among these genes. The expression of both *Dll* and *hth* appears relatively normal in the *ss* null antennal disc (Fig. 2A,B). We therefore conclude that *ss* is not required for either the activation or the maintenance of *Dll* or *hth* expression in the antenna. It has been reported that *Dll* is required for the antennal expression of *ss* (Duncan et al., 1998). To test whether Hth is also required to activate antennal *ss* expression, we examined the effect of ectopic *hth*. We find that ectopic Hth where Dll is expressed, for example in the wing pouch and leg disc, can activate *ss* expression (Fig. 2C-F). Conversely, loss of *hth* in the antenna results in loss of *ss* expression (Ian Duncan, personal communication). Taken together, these results indicate that *ss* functions downstream of both *Dll* and *hth* in the antenna. This led us to test whether *ss* is a primary target through which *Dll* and *hth* function in the antenna.

spalt, *dachshund*, *atonal*, *cut* and *spineless* have antenna-specific patterns of expression

There are only a few genes expressed in either the antenna or the leg but not in both. Among these are *sal* and *salr*, which are identically expressed in a ring pattern in presumptive a2 (Barrio et al., 1999), but are detected at low levels only in leg imaginal disc cells that contribute to the body wall and not to the leg itself (Fig. 3C,G).

In contrast, there are other genes expressed in both antenna and leg precursors that have distinct patterns in the two appendages. Among these are *dac*, *ato*, *ct* and *ss* (Fig. 3A,B,D,E,F,H) (Duncan et al., 1998; Jarman et al., 1995; Mardon et al., 1994). The domain of *dac* expression in the antenna (a3) is much smaller than in the leg where it is expressed in multiple segments (Fig. 3D,H) (Dong et al., 2001). The function of *dac* in antennal development has not been described previously.

The bHLH transcription factor encoding gene, *ato*, is expressed in a ring in presumptive a2, but restricted to small spots in the dorsal leg disc (Fig. 3B,F) (Jarman et al., 1995). *ato* is required for the formation of most chordotonal organs in the fly (Jarman et al., 1993; Jarman et al., 1995). In the antenna, *ato* is required for formation of Johnston's organ (JO) (Jarman et al., 1995), a complex sense organ composed of a large number of chordotonal organs that is used for sensing acoustic vibrations transmitted from the arista through a3 (Eberl, 1999; Goepfert and Robert, 2001).

ct, which is required for differentiation of external sensory (ES) class neurons (Bodmer et al., 1987), is expressed throughout the presumptive proximal antenna (a1 and a2) and head capsule but is expressed in small clusters of cells throughout the leg disc (Fig. 3A,E) (Blochlinger et al., 1993).

ss is expressed in a circular pattern in the antenna covering the presumptive a2 through the arista (Duncan et al., 1998). In the leg disc, *ss* is transiently expressed in a ring pattern in the presumptive tarsal region (Duncan et al., 1998) and subsequently becomes restricted to leg bristle precursors (Fig. 2C) (Duncan et al., 1998). Consistent with the *ss* expression domain, cuticular defects in *ss* null mutants can be found from a2 through the arista. These include the elongation of a2, loss of olfactory sensilla from a3, and transformation of a4, a5, and arista to tarsal segments (Fig. 1D) (Balkaschina, 1929; Burgess and Duncan, 1990; Duncan et al., 1998; Struhl, 1982).

The large differences in the expression patterns of

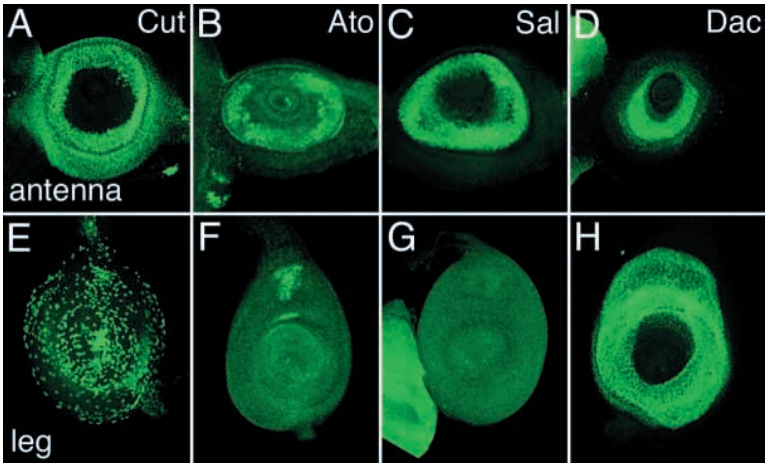

Fig. 3. *cut*, *ato*, *sal* and *dac* are differentially expressed in antenna and leg discs. Wild-type expression of *cut* (A,E), *ato* (B,F), *sal* (C,G) and *dac* (D,H) in wild-type antenna (A-D) and leg (E-F) discs.

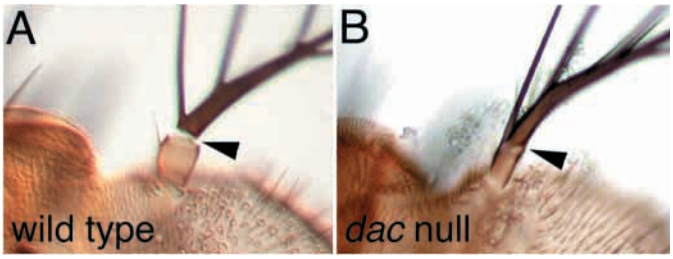

Fig. 4. The a5-arista joint is defective in *dac* null antennae. (A) Wild-type, and (B) dac^3/dac^4 antenna. Arrowheads indicate the location of the a5-arista joint.

these genes between the antenna and the leg led us ask whether these differences are due to differential regulation by antenna-determining genes such as *Dll* and *hth*. To test whether *Dll* or *hth* are responsible for the antenna-specific expression patterns of these genes, we examined the effects on their patterns in *Dll* and *hth* loss-of-function mutants. We also tested whether *Dll* and *hth* are regulating their antenna-specific targets via *ss* by examining their expression in *ss* null antennal discs.

The role and regulation of *dachshund* in the antenna

In contrast to the leg, in the antenna *dac* expression is restricted primarily to a single segment (a3; Fig. 3D,H) (Dong et al., 2001; Mardon et al., 1994). However we can detect trace levels of Dac in areas of the antennal disc immediately distal and proximal to a3 (Fig. 3D and not shown). Because no antennal phenotypes have been reported for loss-of-function *dac* mutants, it was unclear whether *dac* plays a role in patterning this appendage. In transheterozygous *dac* null mutants (dac^3/dac^4), we observe a fusion of the a5 segment with the arista and a reduction in the width of the a5 segment (Fig. 4A,B). This fusion phenotype is similar to what is observed in *dac* hypomorphic and null legs (Mardon et al., 1994). However, unlike the leg phenotype, we find no obvious reductions in length or loss of segments in the *dac* mutant antenna. In addition, we only observe this antennal phenotype in *dac* null animals but not in strong hypomorphic combinations such as dac^{lacZ}/dac^4. Therefore, high levels of Dac are probably not necessary for *dac* function in the antenna.

We previously observed that if Dac levels are elevated in the antenna, expression of *Dll* and *hth* is repressed and medial leg structures are induced (Dong et al., 2001). Therefore if Dac levels are too high, antenna development is compromised. Because *bab* mutants exhibit phenotypes similar to those of *dac*, and *dac* regulates *bab* expression in the antenna (Chu et al., 2002; Godt et al., 1993), we think it likely that antennal *dac* function is mediated via its regulation of *bab*.

We have also found that the antennal *dac* expression domain expands in *Dll* hypomorphs and in *hth* null clones (Dong et al., 2001). This expansion of *dac* expression in *Dll* and *hth* mutant antennae resembles the leg pattern of *dac* expression. In contrast, in the *ss* null antenna, there appears to be neither expansion nor reduction of *dac* expression (Fig. 6G). The only detectable difference in the *ss* null antennal disc is overgrowth in the central (distal) area such that the ring of *dac* expression has a larger radius (Figs 2B and 6G). This correlates with the transformation phenotype of the *ss* null arista into a tarsus, which is a larger structure. Since the expression of *dac* relative to other genes appears normal in *ss* null antennae, we do not think *ss* regulates *dac*.

Regulation of *atonal* in the antenna

The expression of *ato* is required for the formation of the JO (Jarman et al., 1995). The JO is a structure unique to the antenna and is required to sense sound vibrations transmitted from the arista (Eberl, 1999; Goepfert and Robert, 2001). A circular outline of the a2/a3 joint, to which the JO is attached, can be seen in an optical section through the interior of the a2 cuticle (Figs 5A, 6B). This outline is lost in the *ato* null antennae (Fig. 5B). *ato* function is generally associated with neuronal differentiation (Jarman et al., 1993; Jarman et al., 1995), so it is interesting that we observe cuticular defects associated with *ato* null antennae. It may be that formation of the JO is required for the normal morphology of the a2/a3 joint. The circular outline of the a2/a3 joint is lost in *hth* and *Dll* loss-of-function mutants (Fig. 1B,C), but is present in *ss* null mutants (Figs 1D, 5C). Consistent with this, the antennal expression of *ato* is lost in *hth* null clones (Fig. 5F,F′) and in *Dll* hypomorphs (Fig. 5D), but persists in the *ss* null antenna discs (Fig. 5E). Thus although *ss* null mutants exhibit cuticular defects in a2 and a3 (Fig. 1D), the a2/a3 joint to which the JO is attached is present (Fig. 5C). We note that the *Dll* hypomorphic combination used, Dll^{GAL4}/Dll^3, does not lead to loss of a2 (Fig. 7D). Thus the absence of *ato* expression in these antennae is not due to death of the cells that would normally express it.

spalt and *spalt-related* are required for normal development of the second antennal segment

sal and *salr* have similar sequences and are identically

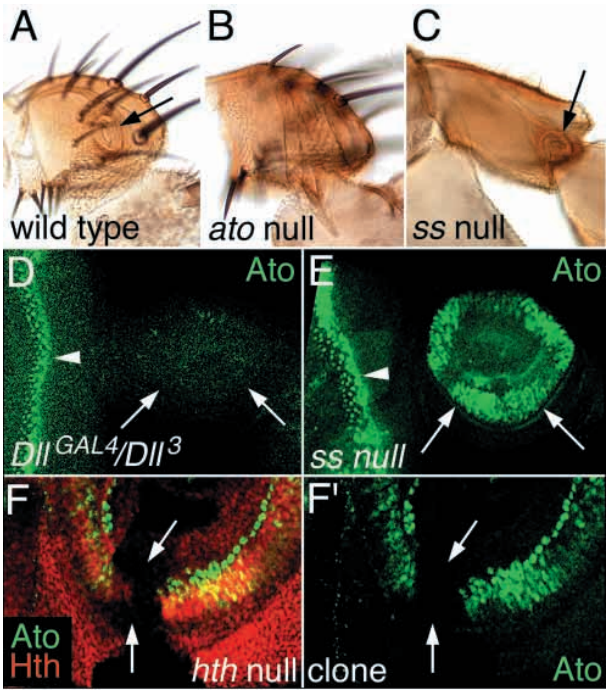

Fig. 5. *ato* is regulated by *Dll* and *hth*, but not *ss* and the a2-a3 joint is defective in *ato* null antenna. (A) Wild type, (B) ato^1 and (C) $Df(3R)ss^{D114.4}/ss^{D115.7}$ second antennal segments (a2). Arrows in A and C indicate the circular outline of the a2-a3 joint which is normal in *ss* mutant antenna, but absent in the ato^1 antenna in B. *ato* expression is lost in a hypomorphic *Dll* antenna (D) and in *hth* null clones (F and F′), but not in a *ss* null ($Df(3R)ss^{D114.4}/ss^{D114.9}$) antenna disc (E). Ato is green in all panels. Hth is red in F. Arrowheads in D and E indicate Ato in the eye.

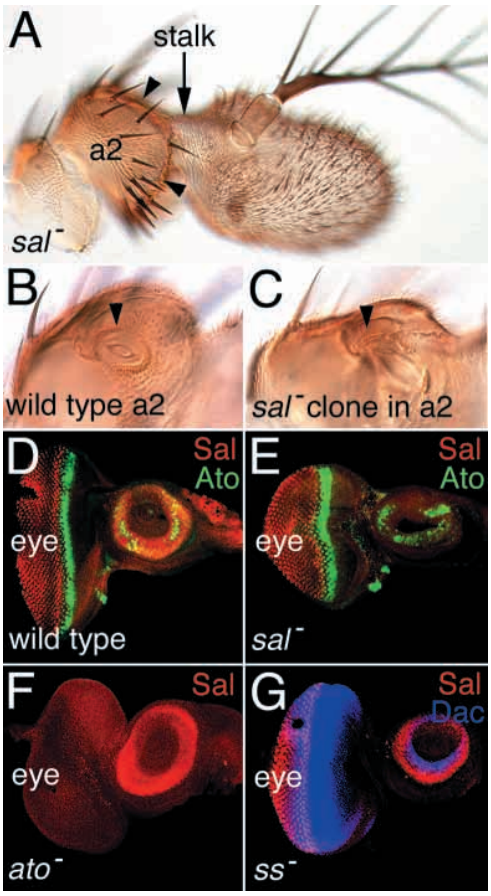

Fig. 6. *sal* mutants exhibit defects in the a2 cuticle and in the a2-a3 joint necessary for audition. **(**A) $sal^{FCK-25}/Df(2L)32FP\text{-}5$ *FRT40A* antenna. a2 is reduced in size distally (arrowheads), exposing the stalk (arrow) of a3 that inserts into a2. (B) Optical section of the interior of a wild-type a2. The circular outline of the a2/a3 joint to which the chordotonal organs of the JO attach is indicated (arrowhead). (C) Optical section of the interior of an a2 in which a *sal/salr* null clone has been induced, and the circular outline of the a2/a3 joint is disrupted (arrowhead). (D) Wild-type expression of Sal (red) and Ato (green) in the eye-antennal disc. (E) Expression of Sal (red) and Ato (green) in a $sal^{FCK-25}/Df(2L)32FP\text{-}5$ *FRT40A* antenna disc. Sal is lacking in the antenna disc, but Ato is still expressed. Note that Sal is expressed normally in the eye disc of this allelic combination. (F) Expression of Sal (red) in an ato^{1} eye-antennal disc. Sal is lost from the eye disc, but expressed normally in the antenna. (G) Sal (red) and Dac (blue) expression in a *ss* null ($Df(3R)ss^{D114.4}/ss^{D114.9}$) eye-antennal disc. Both are expressed normally.

expressed in the antennal imaginal disc in presumptive a2 (Barrio et al., 1999). However, functions for *sal* and *salr* in the antenna have not yet been described. To investigate whether *sal* and/or *salr* are required for normal antenna development, we examined clones null either for *sal* alone or for both *sal* and *salr* in the adult head. Clones null for only *sal* in the antenna have no obvious cuticular phenotypes (not shown). However *Df(2L)32FP-5* clones, which are null for both *sal* and *salr* (Barrio et al., 1999), exhibit cuticular defects in the antenna (Fig. 6A-C). This supports the view that *sal* and *salr* have some redundant functions. The areas affected in the mutants are correlated with their expression domains in the antennal disc. We have also observed a rough-eye phenotype in clones that are null for both *sal* and *salr* but not for *sal* alone (data not shown) (Mollereau et al., 2001).

a2 normally forms a cup, in which a3 sits and must rotate along the PD axis, to transmit sound vibrations from the arista (Goepfert and Robert, 2001). An overall reduction in a2 is observed in $sal^{FCK-25}/Df(2L)32FP\text{-}5$ transheterozygous null antennae (Fig. 6A). In addition, a2 appears to be fused to a3 and a portion of the stalk that connects a3 to a2 is exposed (Fig. 6A). The circular outline of the a2/a3 joint, to which the chordotonal organs of the JO attach, is defective in *Df(2L)32FP-5* clones (Fig. 6B,C) and lost in $sal^{FCK-25}/Df(2L)32FP\text{-}5$ mutant antennae (P. D. S. D., S. Todi, D. Eberl and G. P., unpublished). Furthermore, a3 is unable to rotate in a2. The same antenna phenotypes are observed in sal^{FCK-25} homozygous flies (not shown). However, these phenotypes are not observed in *sal* null clones generated using a sal^{16} *FRT40A* chromosome or in sal^{FCK-25}/sal^{16} transheterozygous antennae (not shown), that do not express *sal* but do express *salr* in the antenna (Barrio et al., 1999). Together, the loss of the a2/a3 joint and the loss of the freedom of rotation of a3 in a2 indicate that *sal/salr* null antennae are defective in hearing and implicate both *sal* and *salr* in normal development of the auditory organ.

Since *ato* is expressed within a subset of the *sal/salr* domain and is activated later than *sal* and *salr* in the antenna, we tested to see whether *Dll* and *hth* activate *ato* via *sal/salr*. We find no detectable reduction of *ato* expression in a2 either in *Df(2L)32FP-5* clones (not shown) or in $sal^{FCK-25}/Df(2L)32FP\text{-}5$ transheterozygous animals (Fig. 6D,E). This allelic combination lacks detectable *sal* and *salr* expression in the antenna, but retains *sal* and *salr* expression in the eye (Fig. 6D,E). The normal expression of *ato* in the antennae of these mutants suggests that the activation of *ato* expression by *Dll* and *hth* is independent of *sal/salr*. Antennal *sal/salr* expression is also unaffected in *ato* null imaginal discs (Fig. 6F). Therefore, *sal/salr* and *ato* are required in parallel for development of antennae that are functional in audition.

We have shown previously that *Dll* and *hth* are required for the expression of *sal* in the antenna (Dong et al., 2000). We report here that *sal* expression does not appear to be affected in *ss* null antenna (Fig. 6G). The fact that Ss is not required for the expression of either *ato* or *sal* in a2 is consistent with our observation that the a2/a3 joint is still present in the *ss* null antenna (Fig. 1D, Fig. 5C).

cut expression in the antennal disc requires *hth*, but not *Dll*

Expression of the homeodomain transcription factor encoded by *ct* almost completely fills the *hth* expression domain of the third instar antennal disc (Fig. 7A,A′). In contrast, the *ct* and *hth* expression patterns in the leg disc are distinct from one another (Fig. 3E and not shown). This made *ct* a strong antenna-specific candidate target for Hth. The antennal expression of *ct* is lost in *hth* null clones (Fig. 7B,B′) indicating that *ct* is indeed downstream of *hth*. To test whether the a2 expression of *ct* also requires *Dll*, we examined *ct* expression in *Dll* mutants. *ct* expression is not reduced in *Dll* null clones or in *Dll* hypomorphs (Fig. 7C,C′,C″,E). Therefore, although *Dll* and *hth* are both required for antennal fate, *cut* is an antenna-specific target of Hth activation that is independent of Dll. As with other antenna-specific targets of Dll and Hth, *ct* expression is also not lost in *ss* null antenna (Fig. 7F).

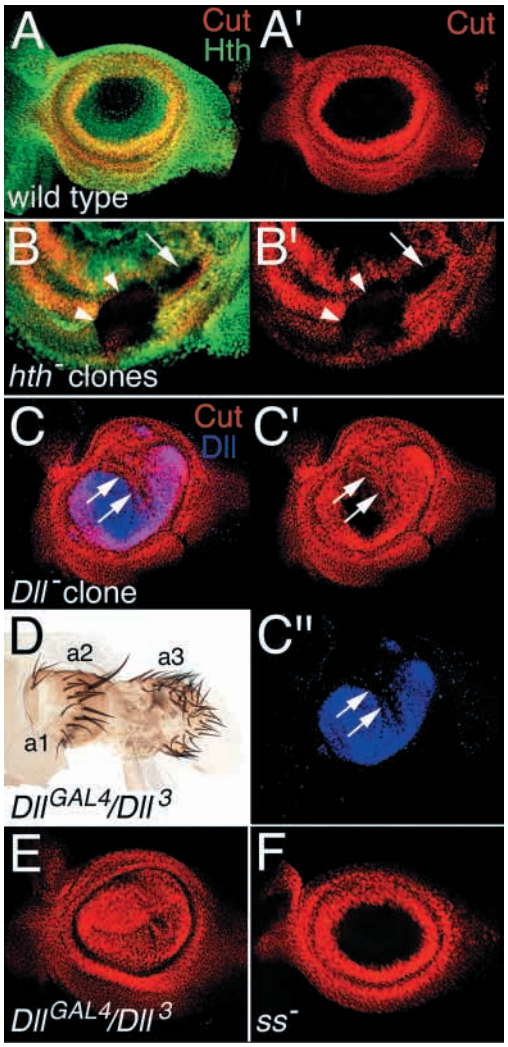

Fig. 7. *cut* expression in the antenna is dependent on *hth*, but not on *Dll* or *ss*. (A,A′). Expression of Cut (red) and Hth (green) in a wild-type antenna disc. (B,B′). Cut (red) expression is reduced (arrowheads) or lost (arrows) in *hth* null clones in the antenna disc. (C,C′,C″) Cut (red) is expressed in a *Dll* null clone in the antenna. Dll is in blue. (D) The allelic combination of Dll^{GAL4}/Dll^{3} results in loss of the arista and transformation of a3 toward leg. a2 is present and has a normal cuticle. (E) Cut (red) is derepressed in the center of a *Dll* hypomorphic antenna disc. (F) Cut (red) is expressed normally in a *ss* null ($Df(3R)ss^{D114.4}/ss^{D114.9}$) antenna disc.

DISCUSSION

Distal-less and *homothorax* regulate multiple targets

Dll and *hth* have dual functions in the *Drosophila* antenna. They are required for the specification of the distal versus proximal domains, respectively, and for the specification of antenna fate. We identify multiple targets of *Dll* and *hth* during antennal development (Fig. 8A). These targets, which have expression patterns unique to the antenna, include *dac*, *ato*, *sal*, *ct* and *ss* (Fig. 8C). Because *ss* mutants also exhibit transformations of the antenna toward leg, and *ss* is a *Dll* target, it was thought that the antenna specification functions of *Dll* and *hth* might occur only via *ss*. Contrary to this, none of the other four genes regulated by *Dll* and *hth* examined here are

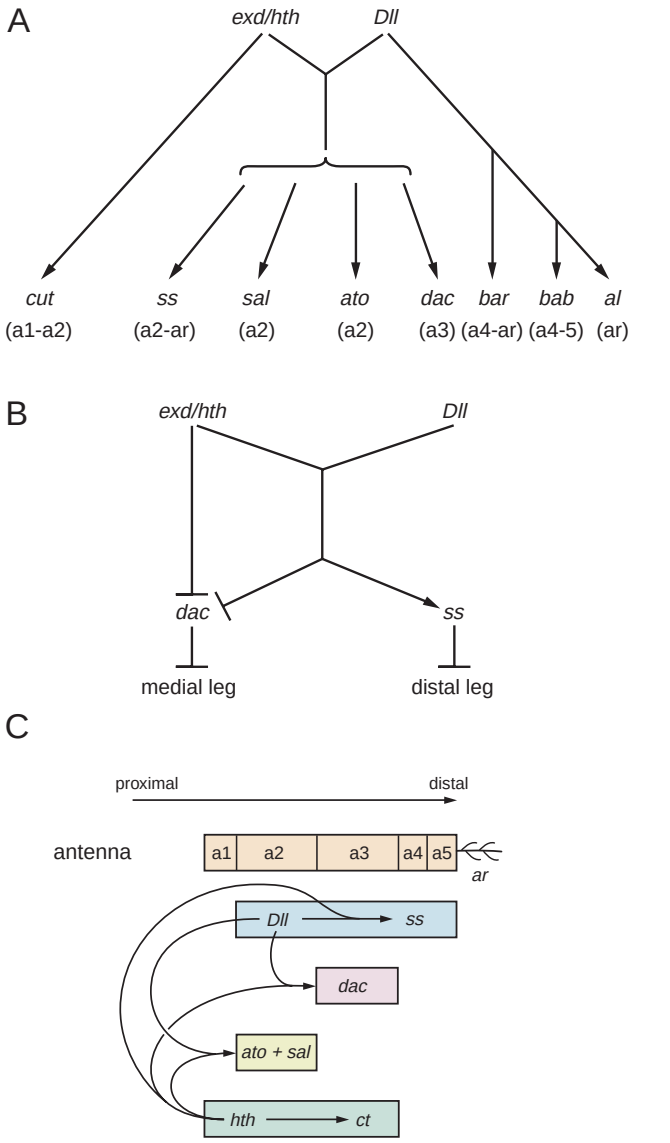

Fig. 8. The genetic hierarchy that governs antenna development. (A) *Dll* and *hth*, probably in conjunction with *exd*, are required for the antennal expression of *ss*, *dac*, *ato* and *sal*, which in turn are required for various aspects of antennal differentiation. *Dll* is required independently for the activation of *arista-less* (*al*), *Bar* and *bab* (Campbell and Tomlinson, 1998; Chu et al., 2002; Gorfinkiel et al., 1997; Kojima et al., 2000), while *hth* is required independently for the activation of *cut*. (B) In addition to activating genes required for specific aspects of antennal differentiation, the homeotic functions of *Dll*, *hth*, *exd* and *ss* also include repression of distinct aspects of leg differentiation. For instance, while *Dll* and *hth* are required for activation of the low level expression of *dac* in a3, they also prevent the high level *dac* expression necessary for medial leg differentiation (Dong et al., 2001). *hth* can also repress *dac* independent of *Dll* since ectopic *dac* expression is observed in *hth* null clones in a1 and the surrounding head capsule (Dong et al., 2001). Since *hth* is required for proximal leg as well as antenna differentiation, *hth* null clones in the proximal antenna are transformed to medial rather than proximal leg structures (P. D. S. D., J. C. and G. P., unpublished observations). *ss* is downstream of *Dll* and *hth* and is required for the repression of distal leg fates (Duncan et al., 1998) (Ian Duncan, personal communication) (this work). (C) Summary of the antennal expression patterns and relationships among *Dll*, *hth*, *ss*, *sal*, *ato*, *dac* and *cut*.

activated via *ss*. We conclude that *Dll* and *hth* regulate multiple independent targets during *Drosophila* antennal development.

The roles of *Distal-less*, *homothorax* and *spineless* in homeotic specification of the antenna

By examining the expression domains, the mutant phenotypes, the genetic interactions and the downstream targets of *Dll*, *hth* and *ss*, we can start to understand the different roles that these homeotic genes are playing in antenna specification. During imaginal disc development, the expression of *Dll* and *ss* is found from a2, a3, a4, a5 and arista. Expression of *hth* is dynamic and retracts from the distal-most segments by late third instar, but *hth* is expressed and cell-autonomously required throughout the antenna from a1 through to the arista (Casares and Mann, 1998).

The *Dll* mutant phenotypes indicate that *Dll* is required both for the distal limb development and for antenna fate. *Dll* hypomorphs exhibit distal limb deletions as well as antenna to leg transformation. The transformation phenotypes of hypomorphic *Dll* antennae can be observed from a2 through to the arista. In these mutants, *hth* expression is not lost or detectably reduced. Thus medial leg structures can develop in the presence of Hth. This suggests that although loss of *hth* expression from the distal and medial leg, via Antennapedia-mediated repression, occurs during normal leg development, loss of Hth is not essential for leg differentiation. It also suggests that the requirement for Antennapedia in normal leg development is not only to regulate *hth*.

Since *ss* is not required to activate antenna-specific expression of genes such as *sal/salr* and *ato* that are involved in antenna differentiation, the question arises as to what *ss* does do in the antenna. As described below, *ss* represses tarsus and tarsal claw organ formation in the antenna. Since loss of *ss* also leads to loss of olfactory sensillae on a3, *ss* probably potentiates the formation of these sensillae, either cooperating with or mediating *Dll* and *hth* activities in a3. Similarly, since ectopic expression of *ss* elsewhere in the body can lead to the formation of ectopic aristae, *ss* may also cooperate with or mediate *Dll* and *hth* activities in arista differentiation.

We demonstrate here that *sal* and *salr*, like *ato*, are required for normal auditory functions. Since both *Dll* and *hth* are required for the antennal expression of *ato* (this work) and *sal* (Dong et al., 2000), *Dll* and *hth* mutant antennae are also hearing defective. In contrast, *ss* null antennae exhibit normal expression of both *ato* and *sal* and normal morphology of the a2/a3 joint, leading us to think that *ss* mutants are likely to be functional in audition.

Homeotic genes act via repression as well as potentiation of tissue fates

We have shown here that homeotic genes, *Dll* and *hth*, regulate multiple targets during antennal development. These targets function in specifying antenna structures and/or in repressing leg development (Fig. 8B). For example, the *ss* mutant phenotype suggests that it represses leg tarsal differentiation. But *ss* is also required for the formation of olfactory sensory sensilla normally found in a3. Although *Dll* and *hth* repress distal leg development via activation of *ss*, their repression of medial leg development appears to be, at least in part, independent of *ss*. Instead, this is achieved via their regulation of the medial leg gene, *dac*, to a narrower domain of expression with lower levels in the antenna as compared to the leg. *sal/salr* and *ato* are required for proper differentiation of a2. However, no transformation phenotypes are associated with the *sal/salr* and *ato* null antenna. This indicates that while *sal/salr* and *ato* are required to make particular antenna-specific structures, they do not appear to repress leg fates. Therefore homeotic genes such as *Dll* and *hth* repress the elaboration of other tissue fates in addition to activating genes required for the differentiation of particular tissues.

The roles of *atonal* and *spalt/spalt-related* in *Drosophila* audition

In third instar imaginal discs, coexpression of Dll and Hth activates *sal/salr* and *ato* in a2 where they, in turn, are needed for JO development. The expression of *ato* is required for the formation of the JO (Jarman et al., 1995) and the a2/a3 joint to which it is attached. Although *sal* and *salr* are not required for the expression of *ato*, the a2/a3 joint is lost in the *sal/salr* null antenna (this work). We expect this leads to improper formation of the JO, although it is also possible that defects in a2/a3 joint formation preclude JO differentiation. In addition, because *sal* is not lost in *ato* null antennae, we conclude that *sal/salr* and *ato* are required in parallel for proper formation of the JO. Furthermore, in the *sal/salr* null antenna, a3 cannot freely rotate within a2. This rotation is necessary for transmission of sound vibrations from the arista to the JO. Taken together, these findings implicate *sal/salr* in *Drosophila* audition. Interestingly, mutations associated with the human homolog of *sal*, *SALL1* cause the human autosomal dominant developmental disorder, Townes-Brocks Syndrome (TBS) (Kohlhase et al., 1998). Auditory defects are characteristic of TBS (Monteiro de Pina-Neta, 1984). Auditory defects are also associated with the human genetic disorder, Split Hand/Split Foot Malformation (SHFM) (Ignatius et al., 1996; Raas-Rothschild et al., 1989), and the SHFM1 locus is linked to the *Dll* homologs, *DLX5* and *DLX6* (Crackower et al., 1996; Scherer et al., 1994). The sensorineural hearing defects associated with the *Distal-less* and *spalt* genes in both *Drosophila* and *Homo sapiens*, in conjunction with a recent finding that *atonal* functions in mouse as well as fly audition (Bermingham et al., 1999; Eberl et al., 2000; Jarman et al., 1993; Jarman et al., 1995), lead us to propose that insect and vertebrate hearing share a common evolutionary origin. We anticipate that further developmental genetic dissection of the *Drosophila* auditory system will provide additional insights into human ear development and suggest that *Drosophila* could provide a useful model system for studying both TBS and SHFM.

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